Development of IgA nephropathy-like glomerulonephritis associated with Wiskott-Aldrich syndrome protein deficiency

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Abstract

Wiskott-Aldrich syndrome (WAS) is a rare X-linked disorder caused by mutations in the *WAS* gene. Glomerulonephritis is a frequent complication, however, histopathological data from affected patients is scarce because the thrombocytopenia that affects most patients is a contraindication to renal biopsies. We found that WASp-deficient mice develop proliferative glomerulonephritis reminiscent of human IgA nephropathy (IgAN). We examined whether increased aberrant IgA production is associated with the development of glomerulonephritis in WASp-deficient mice. Serum IgA and IgA production by splenic B cells was increased in WASp-deficient mice compared to wild-type (WT) mice. A lectin-binding study revealed a reduced ratio of sialylated and galactosylated IgA in the sera from old WASp-deficient mice. Circulating IgA-containing immune complexes showed significantly higher titers in WASp-deficient mice compared to WT mice. These results indicate that the increased IgA production and aberrant glycosylation of IgA may be critically involved in the pathogenesis of glomerulonephritis in WAS.

1. Introduction

Wiskott-Aldrich syndrome (WAS) is a rare X-linked disorder caused by mutations in the WAS gene and is characterized by the triad of thrombocytopenia, eczema, and susceptibility to infection(1). Autoimmune complications are also exceedingly common in WAS and affect 40–70% of patients according to retrospective cohort studies. These associated disorders increasingly complicate the clinical management of WAS as affected patients live longer due to more effective prophylaxis and treatment of infectious complications(2–4). Kidney disease is a frequent complication of WAS. It is found in 3.5–19% of cases(3,5) and can progresses to chronic renal failure, requiring renal transplantation(6,7). However, histopathological data regarding kidney disease is scarce because renal biopsies are often contraindicated due to thrombocytopenia in WAS patients. Membranoproliferative

glomerulonephritis and interstitial nephritis have been reported in a few studies, however, in the majority of cases, IgA nephropathy (IgAN) has been diagnosed(5–11).

IgAN is characterized by immune deposits with dominant or codominant IgA component in the glomerular mesangium(12). Circulating immune complexes (CIC) containing aberrantly *O*-glycosylated IgA1 play a pivotal role in the pathogenesis of IgAN(13–22). It is generally accepted that CIC are formed in IgAN as galactose-deficient IgA1 are produced in affected patients and recognized by anti-glycan IgG or IgA1 antibodies(14,15). In turn, the production of CIC results in deposits in the renal mesangium and glomerular injury.

In WAS patients, increased basal activity of β1,6-*N*-acetylglucosamine transferase (an *O*-linked glycosyltransferase) results in the expression of highly *O*-glycosylated sialophorin or core 2 *O*-glycans(23). Sialophorin is a functional regulator in T-cell activation(24). Therefore, the abnormal *O*-glycosylation of sialophorin may have a role in immune dysfuction of WAS patients(25,26). It has been shown that retroviral-mediated genetic correction can restore the activity of β1,6-*N*-acetylglucosamine transferase and improve glycosylation in lymphoblastoid WAS B-cells(27). Furthermore, aberrant *O*-glycosylation of serum IgA1 with characteristics similar to that observed in IgAN, was detected in a WAS-carrier patient who presented with Henoch-Schönlein purpura(28).

A hyper-IgA (HIGA) mouse, a model for human IgAN with high serum IgA levels, shows reduced galactosylation of *N*-glycans on serum IgA(29). It has been recently reported that NZWxC57BL/6 F1-bcl-2 transgenic mice that are known to develop both IgAN-like and autoimmune lupus-like diseases, show reduced levels of galactosylation and sialylation of serum IgA(30). Altogether, these observations support the notion that aberrant *N*-glycosylation of IgA is involved in the pathogenesis of IgAN.

We have investigated the renal histopathology in 129/SvEv WASp-deficient mice and found that WASp-deficient mice produce autoantibodies and develop proliferative glomerulonephritis with immune complex deposition(31). In this report, we extend our characterization of glomerulonephritis in WASp-deficient mice and evaluated the role of aberrant IgA production in the development of glomerulonephritis in WASp-deficient mice.

2. Methods

2.1 Animals

WASp-deficient (*Was*-KO) mice on a 129 background (129S6/SvEvTac-Was[tm1Sbs]/J) were obtained from Jackson Labs. Mice were maintained in SPF conditions and experiments conducted according to the guidelines of the NHGRI Animal Care and Use Committee.

2.2 Renal histology, immunofluorescence studies, and electron microscopic analysis

Wild-type (WT) control and *Was*-KO mice were euthanized at the specified ages and 5-micron sections of renal tissue were fixed in buffered formalin, stained with periodic acid-Schiff (PAS), and processed for light microscopic evaluation. Specimens that contained >30 glomeruli were used for histopathologic analysis and quantitation of glomeruli with segmental and global sclerosis and/or mesangial cell proliferation and/or an increase in mesangial matrix. Each specimen was assigned a score representative of the calculated percentages of affected glomeruli (0: 0%; 1: 1–24%; 2: 25–49%; 3: >50%). The total maximal score for each specimen was 9 using this scoring system(32). Specimens were evaluated in a triple-blinded manner by 3 nephrologists. Immunofluorescence was performed on four micron cryostat sections of the other cryopreserved kidney with the use of goat anti-mouse FITC-conjugated polyclonal antibodies to IgG, IgA, IgM (K&P Laboratories, Gaithersberg, MD), and rat anti-mouse FITC-conjugated monoclonal antibody

to C3 (Cedarlane, Hornby, Ontario, Canada). For electron microscopic examination, the samples were fixed with glutaraldehyde and osmium tetraoxide, embedded in EPON™ resin. Sections (100 nm) were stained with uranyl acetate and lead citrate, and examined under the electron microscope.

2.3 Measurement of urine albumin, creatinine, and serum IgA

Urine albumin and creatinine were measured using enzyme-linked immunosorbent assay (ELISA) quantitation kits (Alpha Diagnostic International, San Antonio, TX, and R&D Systems, Inc., Minneapolis, MN, USA, respectively), according to the manufacturer's protocol.

Serum IgA was measured using a mouse IgA ELISA quantitation kit, according to the manufacturer's protocol (Bethyl Laboratories, Inc., Montgomery, TX, USA).

2.4 In vitro IgA production of splenic B cells

Resting B cells were negatively selected from splenocytes by immunomagnetic depletion using a mouse B cell isolation kit, (Miltenyi Biotech Inc., Auburn, CA, USA). Cells (1×10^6 cells/mL) were stimulated in 24-well plates with 10 μg/mL lipopolysaccharide (LPS; Sigma, St. Louis, MO, USA) in combination with the following cytokines: 0.5 ng/mL transforming growth factor(TGF)-beta1 (R&D Systems, Minneapolis, MN, USA), 8 ng/mL IL-4 (PeproTech Inc., Rocky Hill, NJ, USA), and 0.25 ng/mL IL-5 (PeproTech). Cells were cultured in RPMI-1640 medium or Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, and1 mM sodium pyruvate. After 4 d of culture, supernatants were collected and tested for IgA levels using a mouse IgA ELISA Quantitation kit (Bethyl Laboratories, Inc.), according to the manufacturer's protocol.

2.5 Detection of circulating IgA-containing immune complexes

Levels of IgA–IgG- or IgA–C3-containing immune complexes in serum were determined by ELISA. Microtiter plate wells were coated with 10 μg/ml anti-mouse IgG (Bethyl Laboratories, Inc.) or 10 μg/ml anti-mouse C3 (MP Biomedicals, LLC, Solon, OH, USA), washed and blocked with bovine serum albumin (BSA; Sigma Chemical Company, St Louis, MO, USA) in phosphate buffered saline (PBS, pH 7.4) containing 0.05%Tween-20 (BSA–PBS–Tween). Serum samples were diluted 100-fold with the same buffer were then applied. After 3 h incubation at room temperature, test samples were removed, wells washed with BSA–PBS–Tween, and incubated for1 h at room temperature with HRP-labelled goat anti-mouseIgA (Bethyl Laboratories, Inc.) diluted 1:100. After washing with the same buffer, wells were incubated with ABTS substrate (Zymed Laboratories Inc., South San Francisco, CA, USA) and optical density (OD) was read at 405 nm.

2.6 Measurement of lectin-binding serum IgA

To examine terminal galactosylation and sialylation of IgA molecules, lectin-binding assays were designed using biotinylated elderberry (Sambucus nigra) bark lectin (SNA; Vector Laboratories, Burlingame, CA, USA) and Ricinus communis agglutinin I (RCA-I; Vector Laboratories), which recognize terminal sialic acid (specifically Neu5Aca2-6Gal) and galactose residues, respectively(29,33). Microtiter plates coated with goat anti-mouse IgA (Bethyl Laboratories, Inc.) were incubated with these samples and biotinylated SNA or RCA-I was then applied. Horseradish peroxidase (HRP)-conjugated streptavidin (Vector Laboratories) was also applied and binding to lectin and IgA were measured. Ratios between of IgA bound to SNA or RCA-I and total IgA were also calculated.

2.7 Statistical analysis

Results are expressed as means ± SD. Statistical significance was determined using the Mann–Whitney test. Differences were considered significant if *P* values were <0.05.

3. Results

3.1 Development of immune complex mediated glomerulonephritis in *Was*-KO mice

Increased mesangial cellularity and matrix deposition with or without endocapillary proliferation were observed in PAS-stained sections from *Was*-KO mice older than 6 months of age, but not in samples from WT control mice (Figures 1A–D). Indirect immunofluorescence showed significantly more intense glomerular IgA, IgG, IgM and C3 complement deposition in the kidneys of WASp deficient mice (Figure 1E–H). Electron microscopic evaluation showed the presence of hump-like deposits, mesangial and paramesangial deposits in *Was-KO* mice older than 6 months of age (Figure 1I, K), while there were no deposits in WT mice older than 6 months of age (Figure 1J). Renal injury score increased in severity with advancing age in *Was*-KO mice and was significantly higher than in WT mice starting at 6 months of age (Figure 2A). Proteinuria in *Was*-KO mice over 6 months of age was significantly elevated compared to WT mice and younger *Was*-KO mice (Figure 2B).

3.2 Elevated serum IgA, increased IgA production of B cells and IgA-containing immune complexes in WASp-deficient mice

Determination of serum IgA levels showed significantly higher titers in *Was*-KO mice compared to WT controls (Figure 3A). Such differences were noted both in young and older *Was*-KO mice. Surface staining of splenic B-lymphocytes showed no differences in the numbers of IgA positive cells between WT and *Was*-KO mice before stimulation (data not shown). After stimulation with LPS, TGF-β1, IL-4, and IL-5 for 4 d, IgA levels in supernatants were significantly greater in *Was*-KO mice compared to WT controls (Figure 3B).

ELISA determination of IgA-containing immune complexes in serum samples from *Was*-KO mice older than 6 month of age showed significantly increased leves of IgA–C3 (Figure 3C) and IgA–IgG (Figure 3D) immune complexes compared to WT littermates.

3.3 Abnormal IgA glycosylation in *Was*-KO mice

Samples from *Was*-KO mice older than 6 month of age contained IgA molecules with significantly lower binding to lectin SNA and RCA-I (Figure 4A–B) compared to WT mice. This abnormality seemed to be age-dependent, as no clear differences in glycosylation were noted among younger WT and *Was*-KO mice.

4. Discussion

IgAN is the most common form of glomerulonephritis in humans and a significant proportion of patients affected with this disorder progress to renal failure(12). Renal disease is a frequent and severe complication of WAS that can lead to kidney failure and consequent need for renal transplantation(3–7). Findings from the limited number of renal biopsies performed in WAS patients have shown variable renal histological features, including membranoproliferative glomerulonephritis and interstitial nephritis. However, in the majority of cases, IgAN has been diagnosed(5–11). Among the speculative mechanisms invoked to explain mesangial IgA deposition in WAS patients, it has been suggested that immune complex deposition and insufficient immune complex clearance may occur after recurrent respiratory infections that often complicate the clinical picture of WAS(8). IgAN,

however, has also been described in X-linked thrombocytopenia, and variant forms of WAS without overt immunodeficiency(32).

Studies in the general population affected with IgAN, have pointed to abnormal features of serum IgA, namely the aberrant galactosylation and sialylation of *O*-glycans in the IgA1 hinge region, as contributory factors to the pathogenesis of the disease(13–22). *O*-linked sugars are composed of *N*-acetylgalactosamine (GalNAc) *O*-linked to serine or threonine residues. The chain may be extended with the addition of galactose to GalNAc and may carry 1 or 2 sialic acids. β1,3 galactosyltransferase catalyzes the transfer of galactose to GalNAc. Defective galactosylation of IgA1 results in increased exposure of GalNAc. It has been reported that IgG–IgA1 and IgA1–IgA1 circulating complexes can form where the IgA1 hinge region *O*-glycans acting as antigenic moiety(13–16). Circulating immune complexes are known to deposit in the renal mesangium and induce glomerular injury; although molecular mechanisms of IgAN are poorly understood, a role of IgA-containing immune complexes cannot be ruled out.

Mouse IgA molecules differ from human IgA for the presence of *N*-glycans instead of *O*-glycans. Human IgA1 has two N-glycosylation sites and several O-glycosylation sites, while mouse IgA has two N-glycosylation sites without O-glycosylation site. Recent evidence has shown that mice lacking β-1,4-galactosyltransferase (β4GalT)-I, which transfers galactose to the terminal *N*-acetylglucosamine of *N*- and *O*-linked glycans, develop glomerular lesions with IgA deposition and expanded mesangial matrix, features that resemble the IgAN pathological findings in humans(35). Also similar to IgAN in humans, β4GalT-I-deficient mice show high serum IgA levels with increased polymeric forms. These findings add to other lines of evidence such as the features of the HIGA mouse model of human IgAN in which high serum IgA levels and reduced galactosylation of *N*-glycans on serum IgA have been demonstrated(29) and the reduced levels of galactosylation and sialylation of serum IgA found in NZWxC57BL/6 F1-bcl-2 transgenic mice that develop both IgAN-like and autoimmune lupus-like diseases(30). These data, together with the findings in human IgAN, indicate that aberrant *N*- or *O*-linked glycosylation of IgA is involved in the pathogenesis of IgAN, both in mice and humans.

Our results show that *Was*-KO mice develop immune complex nephritis. The pathological features and renal impairment observed in these mice are reminiscent of IgAN features reported in WAS(5–11). Our previous investigations have shown intense glomerular IgA, IgG, and C3 complement deposition in the kidneys of *Was*-KO mice compared and the presence of anti-dsDNA and antinuclear antibodies that may play a role in the development of immune complex nephritis in these mice(31). We now show that levels of sialylation and galactosylation of *N*-glycans on serum IgA in *Was*-KO mice are significantly reduced, which support a role of aberrant *N*-glycosylation of IgA in the pathogenesis of immune complex nephropathy that develops in these mice. While in previous studies we have not observed differences in the production of autoantibodies in *Was*-KO mice on the 129SvEv, C3H/HeJ and C57Bl/6J backgrounds(31), we have limited the present study to *Was*-KO mice on the 129SvEv background and we cannot conclude that WASp deficiency would result in similar quantitative and qualitative defects of IgA synthesis in other mouse strains. To this regard, the recent observations by Becker-Herman et al. indicate, in the C57Bl/6J background, severe kidney pathology can develop in the absence of IgA glomerular deposition (36). Further studies are therefore required to reveal how genetic determinants may be associated with increased IgA production and aberrant glycosylation of IgA might in the pathogenesis of glomerulonephritis in 129SvEv Was-KO mice.

Altered oligosaccharide biosynthesis in lymphocytes from WAS patients has been extensively reported(37), however, the specific mechanisms responsible for the aberrant

glycosylation of IgA is unclear. Our results show reduced galactosylation of *N*-glycans on serum IgA only in *Was*-KO mice older than 6 months of age and suggest that the B cell populations that produce aberrant IgA may increase with age. Another possibility is that IgA molecules may be influenced by other factors induced by WASp deficiency, including changes in cytokine balance. Of note, Th2 cytokines are known to affect terminal glycosylation on IgA molecules(33) and Th2 cytokine predominance occurs in *Was*-KO mice(38), which could contribute to the IgA glycosylation defect noted in these mice.

Increased synthetic rate of IgA is a known characteristic of WAS patients(39). In this report, serum IgA levels were increased in 129SvEv *Was*-KO mice regardless of their age and in vitro production of IgA by WASp-deficient splenic B cells was increased compared to WT controls. Further studies are necessary to clarify the mechanisms responsible for the increased IgA production by WASp-deficient B cells, however, these findings may play a role in the pathogenesis of the renal disease observed in 129SvEv *Was*-KO mice, as increased production of abnormally glycosylated IgAs can result in IgA-containing immune complexes and consequent glomerular injury.

In conclusion, we show that 129SvEv *Was*-KO mice produce IgA with defective glycosylation and develop nephritis that is reminiscent of human IgAN, a frequent complication of WAS. Therefore, 129SvEv *Was*-KO mice represent a useful model for the investigation of the role of IgA carbohydrates in the development of IgAN and the physiopathology and of IgAN in WAS, and offer opportunities for developing and testing novel therapeutic strategies.

Acknowledgments

Abbreviations

WAS	Wiskott-Aldrich syndrome
WASp	WAS protein
IgAN	IgA nephropathy
WT	wild type
CIC	circulating immune complexes
HIGA mouse	a hyper-IgA mouse
PAS	periodic acid-Schiff
ELISA	enzyme-linkedimmunosorbent assay
SNA	Sambucus nigra bark
RCA-I	Ricinus communis agglutinin I

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Was-KO >12mo   WT >12mo   Was-KO >12mo
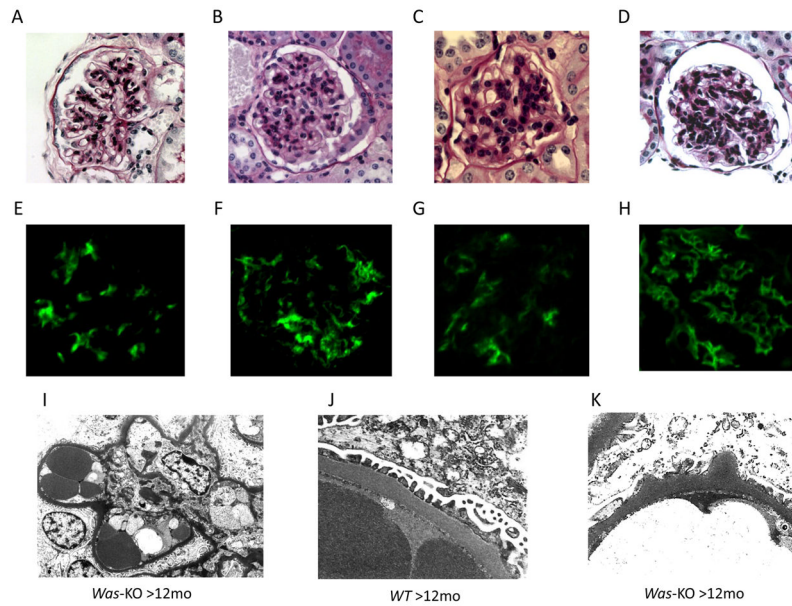
Figure 1. Immune complex deposition and mesangial cell proliferation in WASp deficient mice
Biopsy specimens from WT controls and *Was*-KO mice were examined pathologically at different time points. PAS staining of A: WT (>12months) and B: *Was*-KO (<6months), C: *Was*-KO (6–12months), D: *Was*-KO (>12months). Immunofluorescence studies showed immune complex deposition in *Was*-KO mice. E: IgG, F: IgA, G: IgM, H: C3. Electron microscopic examinations showed mesangial and paramesangial deposits (I), and hump-like deposits (K) in *Was*-KO mice and no deposits in WT mice (J).
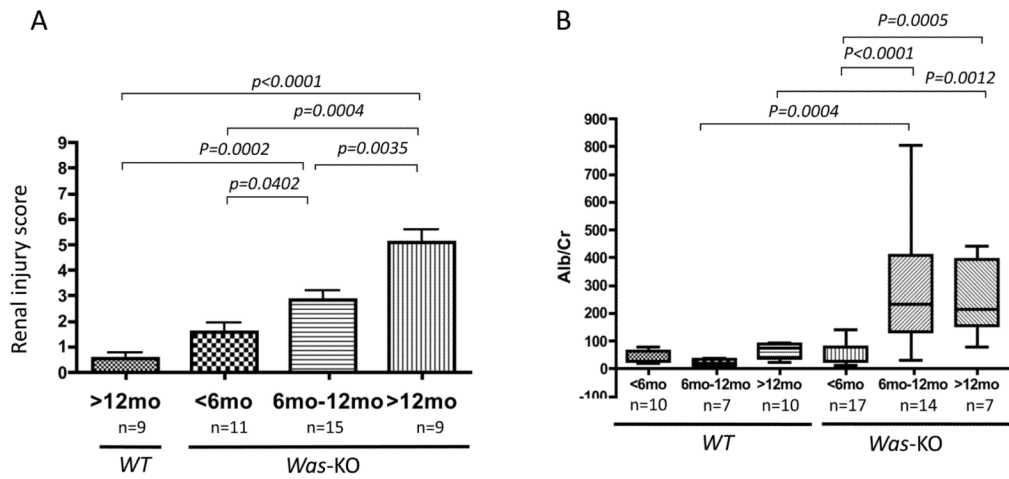
Figure 2. Development of proliferative glomerulonephritis increasing in severity with age
The degree of renal injuries was evaluated with PAS staining and scored (A). The columns represent the mean and bars show SD of samples for each age. Urinary albumin/creatinine ratio was determined in WT and *Was*-KO mice at different time points (B). The bars represent the mean ± SD of samples for each age.
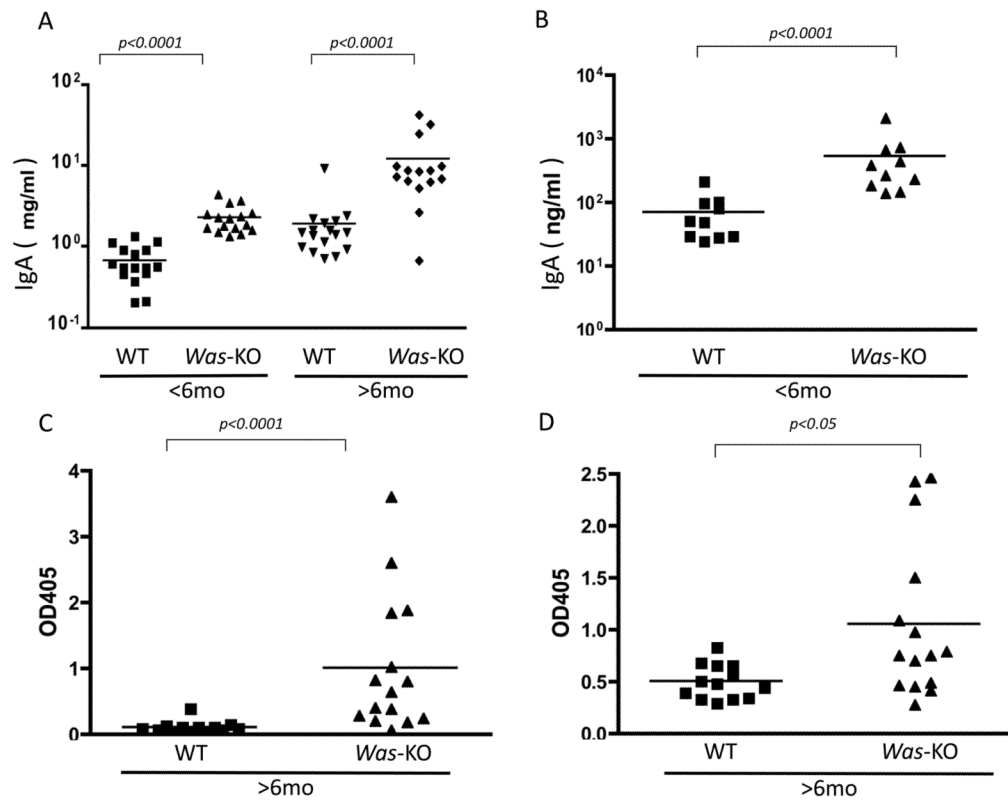
Figure 3. Increased serum IgA, IgA production by splenic B cells and circulating IgA containing immune complexes in WASp-deficient mice

A: Serum IgA; serum IgA was determined by mouse IgA ELISA quantitation kit. Data were compared between WT control and *Was*-KO mice at different age groups.

B: *In vitro* IgA production; splenic B cells were isolated by immunomagnetic isolation kit and the cells were cultured in vitro in the presence of LPS and various cytokines. After 4 d, supernatant samples were harvested and IgA levels determined. Data were compared between WT control and *Was*-KO mice. Circulating IgA containing immune complexes were also determined by ELISA. Data were compared between WT and *Was*-KO mice and expressed as OD′ at 405 nm.

C: Serum circulating IgA-containing immune complex (IgA–C3)

D: Serum circulating IgA-containing immune complex (IgA–IgG)
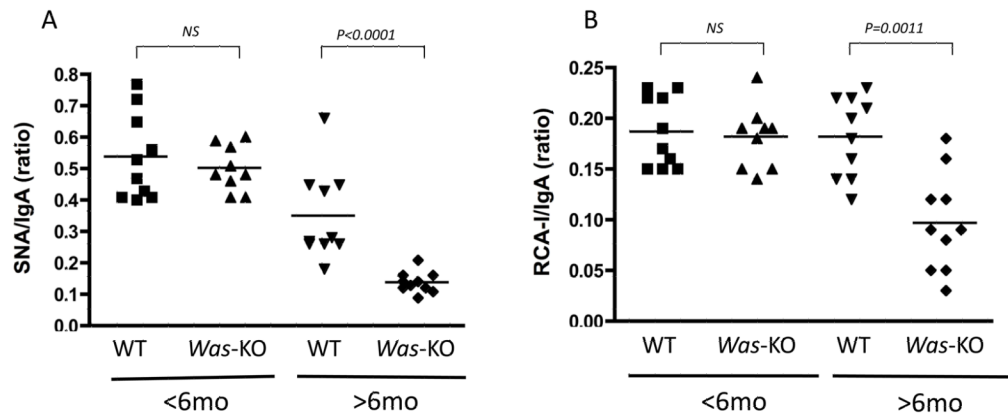
Figure 4. Lectin-binding IgA in sera
Lectin-binding IgA levels were determined by the specific lectin-binding assays as described in Methods. Data were compared between WT control and *Was*-KO mice at different time points. The data are shown as the ratios of specific lectin-binding IgA to total IgA for SNA (A) and RCA-I (B).